

Low-Temperature Damage and Subsequent Recovery of *fab1* Mutant Arabidopsis Exposed to 2°C¹

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The *fab1* mutant of *Arabidopsis thaliana*, which contains increased levels of saturated fatty acids, was indistinguishable from the wild type when it was grown at 22 or 12°C. During the first 7 to 10 d after transfer to 2°C, the growth and photosynthetic characteristics of the *fab1* plants remained indistinguishable from the wild type, with values for the potential quantum efficiency of photosystem II decreasing from 0.8 to 0.7 in plants of both lines. Whereas wild-type plants maintained quantum efficiency of photosystem II at approximately 0.7 for at least 35 d at 2°C, this parameter declined rapidly in the mutant after 7 d and reached a value of less than 0.1 after 28 d at 2°C. This decline in photosynthetic capacity was accompanied by reductions in chlorophyll content and the amount of chloroplast glycerolipids per gram of leaf. Electron microscopic examination of leaf samples revealed a rapid and extensive disruption of the thylakoid and chloroplast structure in the mutant, which is interpreted here as a form of selective autophagy. Despite the almost complete loss of photosynthetic function and the destruction of photosynthetic machinery, *fab1* plants retained a substantial capacity for recovery following transfer to 22°C. These results provide a further demonstration of the importance of chloroplast membrane unsaturation to the proper growth and development of plants at low temperature.

A remarkable feature of the chloroplast membranes of higher plants is the high number of double bonds that are found in the lipid acyl chains. Typically, only about 10% of the fatty acids that compose the hydrophobic mid-portion of the thylakoid bilayer lack double bonds altogether, whereas more than 80% have two or more double bonds (Harwood, 1982). The relevance of thylakoid lipid composition to the correct functioning of these membranes in photosynthesis has been the subject of considerable speculation and experimentation, but it is still not well understood (Quinn et al., 1989; Murata and Wada, 1995).

To investigate the functional significance of chloroplast lipid composition, we have isolated a series of Arabidopsis mutants with specific alterations in leaf lipid composition (Browse and Somerville, 1991; Somerville and Browse, 1991). Five genetic loci have been identified that encode the genes that are involved in lipid-linked fatty acid desaturation of chloroplast lipids (*fad4*, *fad5*, *fad6*, *fad7*, and *fad8*). (The first four of these were previously known as *fadA*, *fadB*, *fadC*, and *fadD*, respectively.) The *act1* mutants are deficient in the chloroplast glycerol-3-phosphate acyltransferase. Each of these mutants exhibits substantial changes in chloroplast fatty acid composition. For example, the *fad5* mutant lacks *cis*-unsaturated 16-carbon fatty acids because of a deficiency in the desaturation of 16:0 on MGD (Kunst et al., 1989a), whereas the *fad6* mutant has increased levels of monoenoic fatty acids as a result of a mutation in the chloroplast 16:1/18:1 desaturase (Browse et al., 1989). However, the mutants do not exhibit a visible phenotype under normal conditions, and when grown at 22°C (and continuous light at 100–150 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ PAR) exhibit only very minor effects on growth, net CO₂ fixation, photosynthetic fluorescence, and photosynthetic electron transport. Instead, the most pronounced effects are on the size of the chloroplasts (McCourt et al., 1987) or the organization of the thylakoid membranes (Hugly et al., 1989; Kunst et al., 1989b).

More clear-cut consequences on the biology of mutant plants are observed for some of the mutants at higher (Hugly et al., 1989; Kunst et al., 1989c) or lower temperatures. The *fad4*, *fad7*, *fad8*, and *act1* mutants all grow as well as the wild type at 5°C, but *fad5* and *fad6* plants show partial chlorosis and a 20 to 30% reduction in their growth rate compared with wild-type plants at this temperature (Hugly and Somerville, 1992). Ultrastructural analysis of chloroplasts from plants grown at a low temperature indicated major reductions in the amounts of both appressed and nonappressed thylakoids and concomitant reductions in the amounts of Chl and membrane glycerolipids. These

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Abbreviations: Chl, chlorophyll; F_m , maximal fluorescence; F_m' , maximum fluorescence under actinic illumination; F_v , variable fluorescence; F_v/F_m , maximum quantum yield of PSII photochemistry; MGD, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; ϕ_{II} , quantum yield of noncyclic electron transfer or steady-state quantum efficiency; SL, sulfoquinovosyldiacylglycerol; X:Y, a fatty acyl group containing X carbon atoms and Y double bonds (*cis* unless specified); double-bond positions are indicated relative to the carboxyl end of the chain (e.g. $\Delta 12$) or the methyl end of the chain (e.g. ω -6).

results suggest that the changes in the fatty acid composition in the *fad5* and *fad6* mutants impair chloroplast development at low temperatures. Notwithstanding these observations, both the *fad5* and *fad6* mutants continue to grow and complete their life cycles normally at 6°C. These mutants demonstrate that chloroplast membrane unsaturation can be a factor that affects the low-temperature responses of plants. However, although the phenotypes that have been described show some parallels to the physiology of chilling-sensitive plants, a detailed characterization of the mutants does not recommend that these mutants be used as models for the processes involved in chilling injury (Hugly and Somerville, 1992).

One of the most enduring hypotheses in the general area of a plant's susceptibility to chilling stress proposes that the molecular species of chloroplast PG, containing a combination of saturated fatty acids (16:0, 18:0, or 16:1-*trans*) at both the *sn*-1 and *sn*-2 position of the glycerol backbone (high-melting-point PG molecular species), confer chilling sensitivity to plants (Murata and Yamaya, 1984; Murata et al., 1992). Although this hypothesis has been supported by a number of experimental observations, the recent characterization of the *Arabidopsis fab1* mutants has provided a counterexample that demonstrates that the high-melting-point species of PG can have no more than a contributory effect in inducing chilling sensitivity. PG from the leaves of *fab1* plants contained 43% high-melting-point molecular species, a higher percentage than is found in approximately one-half of the chilling-sensitive plants that have been examined. Nevertheless, the mutant was completely unaffected (when compared with wild-type controls) by a range of low-temperature treatments that quickly resulted in the death of known chilling-sensitive plants. These treatments included up to 7 d at 2°C, either in the dark or under 150 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ of light, and freezing at -2°C for 24 h (Wu and Browse, 1995).

Even though the *fab1* plants were not damaged by these chilling and freezing treatments, we did find that growth of the mutant was severely affected when plants were grown for extended periods (>2 weeks) at 2°C (Wu and Browse, 1995). This long-term damage appears to be a key to understanding the importance of saturated and unsaturated fatty acids in chloroplast function at low temperature. We have, therefore, monitored photosynthetic fluorescence parameters and other characteristics of *fab1* and wild-type plants during 35 d of exposure to 2°C and during recovery. Our results indicate that the *fab1* plants are dramatically affected by the low-temperature treatment. During 3 to 4 weeks they suffered an almost complete loss of photosynthetic function and an associated destruction of chloroplasts within leaf cells. The mutants also demonstrated a remarkable capacity for recovery when they were returned to 22°C.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The wild-type line of *Arabidopsis thaliana* (L.) Heynh. used in this study is the Columbia ecotype, and the mutant

line 1A9, which carries a mutation at the *fab1* locus, was derived from this ecotype following mutagenesis with ethyl methanesulfonate as previously described (James and Dooner, 1990). The mutant was backcrossed to the wild type four times before being used for the experiments described here.

Plants were germinated and grown on a commercial potting mixture at 22°C under continuous fluorescent illumination (150 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$). Continuous illumination reduces complications associated with diurnal variations in physiology and biochemistry and allows for rapid growth of the plants. After 11 or 14 d, the seedlings were transferred to controlled environment chambers at 12 and 2°C under continuous fluorescent illumination (150 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$). For recovery experiments, plants were returned from 2 to 22°C and at different times.

Measurements of Growth Rate

Plants were germinated at 22°C and grown at standard growth conditions as described above. After 11 d, one set of plants was transferred to 2°C under constant illumination (150 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and the second set was transferred to 12°C under the same illumination. The third set was kept at the standard growth conditions. Samples of five plants were harvested at 3-d intervals for the next 12 to 30 d, and the fresh weights of the aerial portions were measured.

Extraction and Analysis of Lipids

Leaves were harvested and their fresh weights were determined before grinding them under liquid N₂. The lipids were extracted with chloroform:methanol:formic acid (10:10:1, v/v). After the sample was centrifuged, the supernatant was decanted and the tissue re-extracted with chloroform:methanol:water (5:5:1, v/v; Browse et al., 1986). The extracts were combined and washed with 0.2 M H₃PO₄/2 M KCl (Hajra, 1974). Lipids were recovered in the chloroform phase, dried under N₂, and taken up in a small volume of chloroform.

Lipids were separated by TLC on (NH₄)₂SO₄-impregnated silica gel plates with the solvent system of acetone:benzene:water (30:10:2.7, v/v; Khan and Williams, 1977). For quantification and fatty acid composition analysis, lipids were transmethylated with 2.5% H₂SO₄ in methanol after the addition of 17:0 methyl ester as an internal standard. The resulting methyl esters were then quantified by GC (Browse et al., 1986).

Chl Measurements

Chl content was determined in 80% acetone spectrophotometrically by measuring the A_{645} and A_{663} . Then, it was calculated by following formulas: $\mu\text{g Chl} = (20.2 \times A_{645}) + (8.02 \times A_{663})$ or $\mu\text{g Chl } a = (12.7 \times A_{663}) - (2.69 \times A_{645})$ and $\mu\text{g Chl } b = (22.9 \times A_{645}) - (4.68 \times A_{663})$ (Arnon, 1949).

Measurements of Chl Fluorescence from PSII

Measurements of modulated Chl fluorescence were made with a pulse amplitude modulation fluorometer

(model 101, Heinz Walz, Effetrich, Germany), and the values of F_v/F_m were determined in the dark at 22°C. Plants were removed from 2°C and dark-adapted at 22°C for 1 h. Dark-adapted leaves were exposed to the weak, modulated measuring beam with a PPFD of $0.025 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ to determine the instantaneous fluorescence. F_m was measured with a saturating pulse (400 ms duration) and a PPFD of $4500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The difference between F_m and instantaneous fluorescence is termed the F_v . To measure steady-state quantum efficiency, plants were exposed to actinic light with a photon flux density of $140 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ and allowed to reach steady-state photosynthesis. The steady-state value of fluorescence, F_s , was then determined, followed by a saturating pulse to determine F_m' . The ϕ_{II} was then calculated as $\phi_{II} = (F_m' - F_s)/F_m'$ (Genty et al., 1989).

Electron Microscopy

The leaves were cut into small squares in 3% glutaraldehyde in 0.1 M Pipes buffer (pH 7.2) at 4°C. Then, they were fixed in the same solution overnight at 4°C. After they were washed in 0.1 M Pipes (pH 7.2) 4 times for 10 min each time, they were put into 2% OsO_4 overnight at 4°C for secondary fixation. The specimens were washed in 0.1 M Pipes (pH 7.2) again and dehydrated through a graded ethanol series as well as a graded acetone series. Next, they were embedded in Spurr's epoxy resin (Sigma). Thin sections were stained with uranyl acetate and lead citrate and examined in an H300 transmission electron microscope (Hitachi, Danbury, CT).

RESULTS

Reduced Growth of *fab1* Is Observed Only after 2 Weeks at 2°C

To quantify the overall effect of low-temperature growth on *fab1* plants, we grew wild-type and mutant plants for 11 d at 22°C, a continuous photon flux density of $150 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, and 60 to 70% RH, and then transferred samples of plants to 12 or 2°C under the same growth conditions. At the time of transfer, the seedlings had two true leaves and an average shoot fresh weight of 12.8 ± 0.2 mg per plant. As shown in Figure 1A, growth of *fab1* controls at 22°C, measured as the increase in shoot fresh weight, was indistinguishable from the wild type at this temperature, with a relative growth rate (ω^{-1}) of 0.416. After transfer

to 12°C, the growth of both the mutant and wild-type plants was slowed but there was essentially no difference between the relative growth rates of the two lines (Fig. 1B) ($\omega^{-1} = 0.199$ for *fab1*; $\omega^{-1} = 0.190$ for wild type). At this temperature, as well as at 10°C (Wu and Browse, 1995), the *fab1* mutant completed its life cycle as quickly as the wild type and produced abundant, viable seeds.

Wild-type Arabidopsis plants were not injured by the exposure to lower, nonfreezing temperatures and continued to grow and develop during prolonged exposure to 2°C (Fig. 1C). Although the relative growth rate of wild-type plants ($\omega^{-1} = 0.034$) was an order of magnitude lower than at 22°C, the plants exhibited normal morphology and appearance. During the first 12 to 15 d following transfer to 2°C, the fresh weight gains of the *fab1* mutant were entirely similar to those of the wild type (Fig. 1C). However, beyond 15 d of growth the mutant slowed dramatically. By 21 d, leaves of the *fab1* plants were noticeably chlorotic and the plants were no longer increasing in fresh weight.

Effects of Low Temperatures on Membrane Lipids and Chl

The *fab1* mutant was previously characterized as having increased proportions of 16:0 in all of the major membrane lipids of the leaf tissue (Wu et al., 1994). The increased proportion of 16:0 in PG of *fab1* chloroplasts suggested that the mutant might contain elevated levels of high-melting-point PG, and this was confirmed by separation and quantitation of the PG molecular species (Wu and Browse, 1995). In the experiments described here we extended this characterization by sampling leaf material from wild-type and mutant Arabidopsis plants before they were transferred to 2°C (0 d), and at 7-d intervals during the 2°C treatment. The most relevant comparisons were between plants sampled at 0 d and those sampled at 14 d when growth and photosynthesis of *fab1* plants (see below) were beginning to be severely affected. The data from these analyses are included in Table I. There were a number of changes in the lipid composition that occurred in both wild-type and mutant leaves during the 14-d period. The overall level of lipid unsaturation increased, mainly because the proportion of 18:3 (16:3 in MGD) increased, there was a large reduction in 16:1-*trans* in PG and a concomitant increase in 16:0, and the proportion of chloroplast lipids (MGD plus digalactosyldiacylglycerol plus PG plus SL) decreased from 63 to 54% of the total leaf lipids in both the wild-type and *fab1* plants. By all of these criteria, the lipid

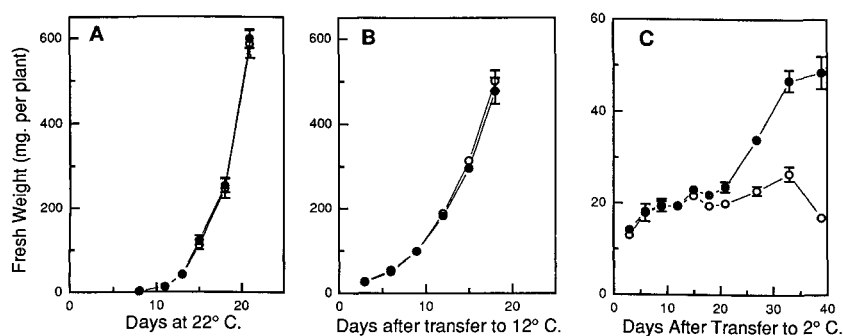


Figure 1. Growth curves for wild-type (●) and *fab1* (○) mutant Arabidopsis at 22°C (A), 12°C (B), and 2°C (C). Growth measurements were made by taking the fresh weight of the entire aboveground portions of plants at the indicated intervals. Values shown are the means \pm SD ($n = 5$).

Table 1. Fatty acid compositions of wild-type and *fab1* mutant plants prior to transfer to 2°C (0 d) and after 14 d at 2°C

Plants were 11 d old at the start of the 2°C treatment. The plants sampled were from the same experiment used to obtain the data in Figures 2 to 4.

Fatty Acid Composition	MGD		DGD ^a		PG		SL		PC ^b		PE ^c		PI ^d	
	0 d	14 d	0 d	14 d	0 d	14 d	0 d	14 d	0 d	14 d	0 d	14 d	0 d	14 d
Wild type														
16:0	1.7	3.9	13.6	11.1	24.8	38.4	36.7	37.6	20.2	18.3	28.5	22.7	41.6	42.9
16:1	1.3	— ^e	—	—	25.4 ^f	9.1 ^f	—	—	0.8	—	—	—	—	—
16:3	31.4	37.2	2.8	4.0	—	—	—	—	—	—	—	—	—	—
18:0	—	—	1.1	—	1.3	0.7	5.4	—	2.6	0.7	2.8	0.6	3.0	1.0
18:1	1.4	0.6	1.5	0.6	6.5	2.8	5.4	—	7.2	4.5	4.4	2.4	2.8	1.7
18:2	3.9	1.9	5.5	2.9	10.4	9.5	17.3	10.0	42.2	31.9	41.0	34.7	22.5	19.1
18:3	57.3	54.8	74.4	80.4	31.3	38.9	35.2	54.4	26.7	43.6	23.0	39.0	29.9	34.9
Proportion of leaf polar lipids	35.9	27.6	14.3	15.5	10.3	9.7	2.1	1.6	19.6	24.3	11.6	15.5	6.3	5.9
<i>fab1</i> mutant														
16:0	2.9	7.8	18.6	16.0	39.3	56.1	44.3	37.0	27.0	27.6	33.0	22.6	47.6	46.2
16:1	1.1	1.0	1.3	0.9	25.5 ^f	9.9 ^f	—	—	2.9	2.9	1.6	1.6	—	0.7
16:3	37.7	41.1	3.8	6.5	—	—	—	—	—	—	—	—	—	—
18:0	—	—	0.8	—	1.2	—	—	1.8	2.0	1.0	1.6	—	1.6	0.9
18:1	1.4	—	2.5	1.8	3.7	2.4	—	2.3	6.2	3.4	4.7	7.2	2.2	2.1
18:2	2.2	1.2	4.0	2.5	7.4	3.6	10.1	10.9	29.2	11.4	30.2	14.4	15.1	8.5
18:3	51.4	46.9	68.2	71.6	22.5	26.2	41.0	40.7	30.9	51.9	28.0	52.4	33.0	40.2
Proportion of leaf polar lipids	40.0	30.4	10.5	10.4	10.7	11.8	1.3	1.2	18.7	23.0	11.9	15.9	6.8	7.4

^a DGD, Digalactosyldiacylglycerol.
tol. ^e —, Acyl group was not detected.

^b PC, Phosphatidylcholine.
^f $\Delta 3$ -trans isomer.

^c PE, Phosphatidylethanolamine.

^d PI, Phosphatidylinositol.

changes associated with low-temperature incubation were very much the same in the mutant and wild type. Equally important was our observation that the major difference in lipid composition between *fab1* and wild type was unchanged by 14 d at 2°C; the lipids from *fab1* leaves retained an increased proportion of 16:0 relative to the wild type.

Between the 14-d sampling time and the termination of the experiment at 35 d, there were further modest changes in lipid composition (data not shown). In the wild type the proportion of chloroplast lipids remained approximately 54% of the total leaf lipids, but in *fab1* the proportion of chloroplast lipids decreased gradually to 45% of total leaf lipids at 35 d. This decrease in chloroplast lipids, together with changes in the fatty acid compositions of individual lipids from *fab1* leaves, accompanied the cessation of growth and progressive chlorosis of the plants during incubation at 2°C (Wu and Browse, 1995; Fig. 1C).

To more accurately describe the changes that accompanied the visual chlorosis of *fab1* plants during long-term exposure at 2°C, we measured the Chl contents of *fab1* and wild-type plants during the course of the experiment. Before transfer to low temperature, wild-type and *fab1* plants contained 1.3 to 1.4 mg total Chl g⁻¹ fresh weight, and the Chl content of the control plants that was left at 22°C remained in the range of 1.2 to 1.5 mg g⁻¹. After the plants were transferred to 2°C, the Chl content of the wild type decreased steadily during the first 14 d (Fig. 2) but then increased again and reached 1.26 mg g⁻¹ by the end of the experiment. During the first 14 d at 2°C, the modest decline in the Chl content of the *fab1* leaves matched changes in the wild type, and it was only after this time that Chl in the mutant decreased sharply.

Drastic Reductions in Photosynthetic Parameters in the *fab1* Mutant

Fluorescence analysis of plants grown at 22°C and the moderate light levels used for Arabidopsis plants in this study (150 μ mol quanta m⁻² s⁻¹) have not revealed any significant difference between the *fab1* mutant and wild-type Arabidopsis (J. Wu, N. Warwick, and J. Browse, unpublished data). To monitor the relative effects of low-temperature growth on mutant and wild-type plants, we

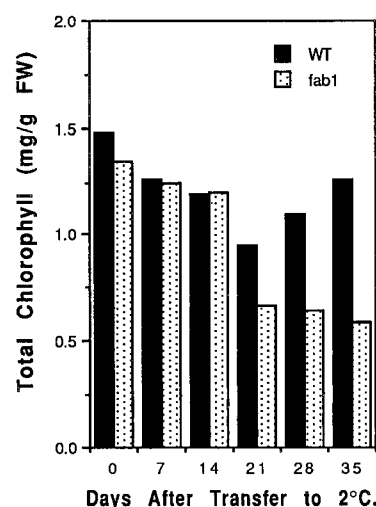


Figure 2. Chl content of wild-type (WT) and mutant (*fab1*) Arabidopsis plants. Plants were germinated, grown for 14 d at 22°C, and then transferred to 2°C for 5 weeks. FW, Fresh weight.

measured dark F_v/F_m and ϕ_{II} at intervals after transfer of the plants to 2°C. The time course of F_v/F_m (Fig. 3) showed a modest decrease in both the mutant and wild type during the first 7 d of the experiment, but after this time F_v/F_m for *fab1* declined dramatically and the value of this parameter was less than 0.1 at 21 d. In contrast, F_v/F_m for the wild type stabilized at a value of approximately 0.7. Measurements of the quantum yield closely paralleled the results shown in Figure 3 for both the mutant and wild-type samples (data not shown), suggesting that the substantial collapse of photosynthesis that occurred in *fab1* plants was largely the result of a severe disruption of the photosystems (or at least of PSII) and was probably not preceded by any major effects on other aspects of the photosynthesis process.

Specific Disruption of Chloroplasts during Exposure of *fab1* Plants to Low Temperature

When mutant and wild-type plants were grown at 22°C, they were not only indistinguishable in growth (Fig. 1) and morphology but also in their cellular ultrastructure. The transmission electron micrographs in Figure 4, A and B, show typical views of wild-type and *fab1* chloroplasts from plants grown at 22°C. Although quantitative morphometric analysis was not carried out, these and other micrographs indicate that there were no major differences in the organization or structure of the granal and stromal thylakoids or in other aspects of chloroplast appearance.

After 7 d at 2°C, the chloroplasts in the mesophyll cells of wild-type and mutant leaves looked similar to the views shown in Figure 4, A and B (micrographs not shown). Chloroplasts from *fab1* still contained numerous large starch grains, as did the wild-type chloroplasts. By 14 d, however, a dramatic change in ultrastructure had taken place in the mutant. Whereas chloroplasts of the wild type (Fig. 4C) remained normal in appearance, those of the mutant were now irregular in shape with poorly defined or

broken envelopes (Fig. 4D). The accumulated starch had entirely disappeared and the thylakoids were reorganized into extremely long regions of appressed membranes interspersed with nonappressed membranes. The proportion of nonappressed membranes appeared to be increased relative to the chloroplasts from *fab1* plants grown at 22°C. The disrupted chloroplasts were always associated with small membrane-bound vacuoles, which seemed to be at the periphery of the now disorganized chloroplasts. Apart from these vacuoles, the major ultrastructural changes were largely confined to the chloroplasts. The mitochondria, vacuolar and tonoplast membranes, ER, and nuclei of the cells remained substantially unaffected in appearance. Some of these features are evident in Figure 4D.

After 21 d at a low temperature, chloroplasts had largely disappeared from the mutant (Fig. 4F). Some remnants in the form of appressed and nonappressed membranes were visible in most mesophyll cells, but clearly the ultrastructure reflects the almost complete loss of photosynthetic function indicated by the fluorescence data. Even with this level of chloroplast degeneration, the appearance of other structures in the *fab1* cells was remarkably normal; for example, three mitochondria appear in Figure 4F.

Damaged *fab1* Leaves Retain a Substantial Capacity for Recovery

We previously reported the overall appearance of the *fab1* plants maintained at 2°C for 28 d and the capacity of these same plants to recover and subsequently complete their life cycle upon transfer to 22°C (Wu and Browse, 1995). However, after reviewing the transmission electron micrographs shown in Figure 4, we could not accept that extensive recovery of photosynthetic function at 22°C could be possible in the short term. To investigate recovery, we repeated the experiment described in Figure 3, except that after 21 d at 2°C and again after 35 d three pots each of the wild-type and *fab1* plants were returned to 22°C. The individual leaves upon which measurements of F_v/F_m and ϕ_{II} were made at 21 d were marked and then resampled at intervals after the plants were returned to 22°C. Thus, recovery of photosynthetic fluorescence parameters was monitored on a single set of leaves for the duration of each low-temperature treatment.

The moderate chlorosis observed in *fab1* plants after 21 d of chilling (Fig. 5, A and B) was heightened appreciably within the first 48 h after return to 22°C (Fig. 5, C and D), even though F_v/F_m measured on nearly mature leaves of these plants (Fig. 5, arrows) had recovered considerably after this time (Fig. 6). Between 2 and 4 d after return to 22°C, the exposed leaves did become darker green, although they remained pale relative to wild-type controls and to new leaf tissue developing on the *fab1* plants (Fig. 5, E and F). The period from 2 to 4 d also saw a further increase in F_v/F_m to a value of 0.62 in these same leaves (Fig. 6). Even when F_v/F_m had decreased to 0.02 after 35 d at 2°C, a shift to 22°C was followed by a steady increase in the value of this parameter over several days to 0.51 after 7 d of recovery (Fig. 6). Throughout the damage and recovery phases of this experiment, the quantum yield of

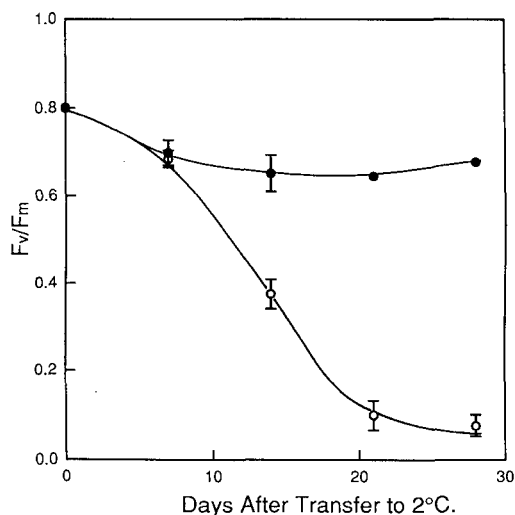


Figure 3. The F_v/F_m of PSII from wild-type (●) and mutant (○) *Arabidopsis* plants grown at 2°C. Plants were germinated, grown for 14 d at 22°C, and then transferred to 2°C for 5 weeks. Values shown are the means \pm SD ($n = 5$).

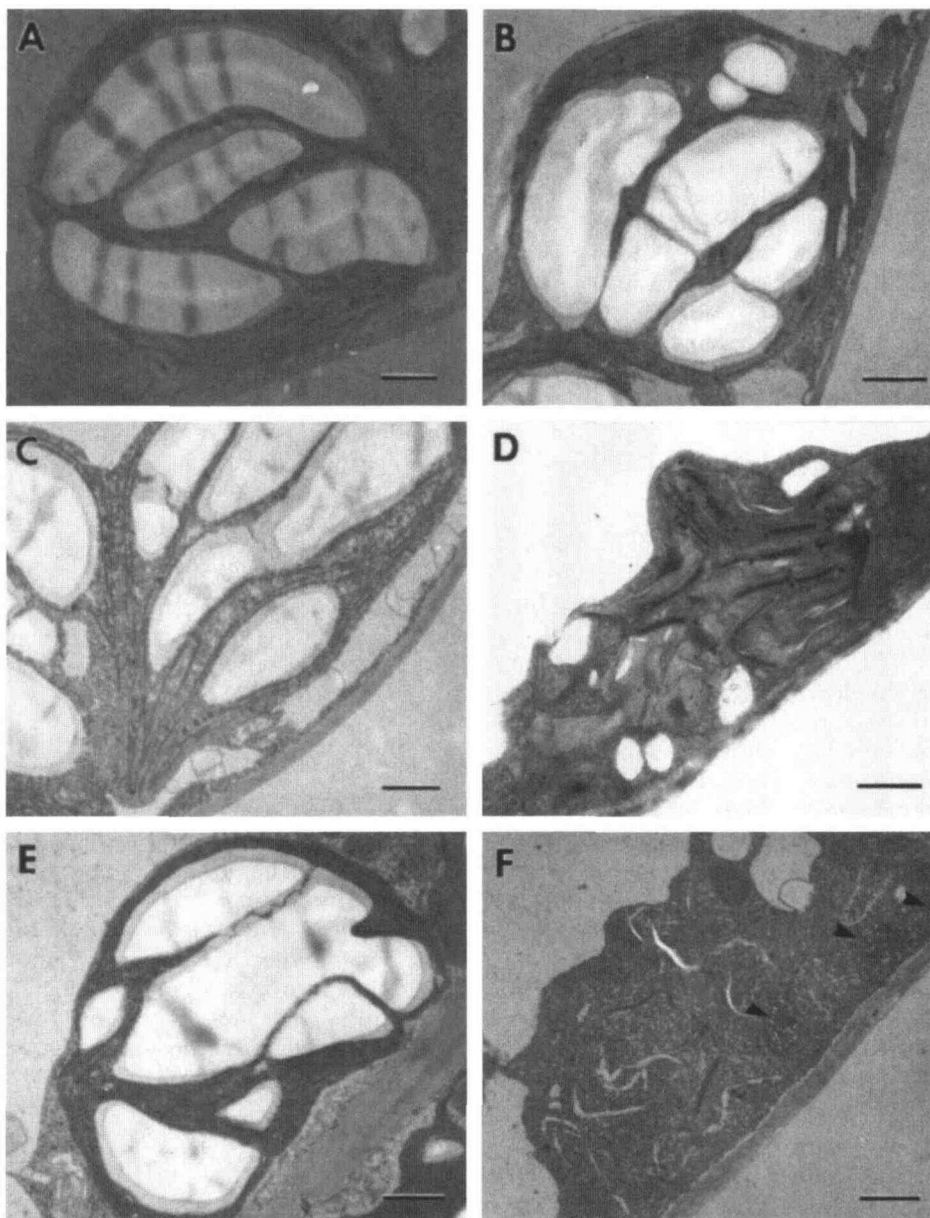


Figure 4. Transmission electron micrographs of chloroplasts from wild-type and mutant rosette leaves of *Arabidopsis*. A and B, Wild type and *fab1* grown at 22°C; C and D, wild type and *fab1* grown at 2°C for 14 d; and E and F, wild type and *fab1* grown at 2°C for 21 d. Bar $\approx 1 \mu\text{m}$. Arrowheads in F indicate mitochondria.

photosynthesis closely paralleled the decrease and increase of F_v/F_m (data not shown), suggesting that the results in Figure 6 reflect the actual performance of photosynthetic centers in the leaves that were monitored.

For both sets of *fab1* plants (those monitored for recovery after 21 and 35 d at 2°C), substantial increases in F_v/F_m were measured before any appreciable greening of the leaves had occurred. Following 21 d of low-temperature treatment and 7 d at 22°C, F_v/F_m recovered to nearly 90% of the value in untreated controls but the leaves remained chlorotic (not shown). These findings reflect the fact that fluorescence measurements determine the status of the photosystems that are present rather than the overall photosynthetic rate on a leaf area basis. The chlorosis of *fab1*

leaves presumably reflects a net loss of photosynthetic machinery, but the reduced number of photosystems that are present have, nevertheless, regained a high level of efficiency.

DISCUSSION

A New Low-Temperature Phenotype and Possible Chloroplast Autophagy in *fab1* Plants

The distinctive changes in physiology and cell ultrastructure that we have observed in *fab1* plants exposed to 2°C represent a case history that is quite different from those described for other plant lines with altered membrane lipid



Figure 5. The appearance of wild-type and mutant *Arabidopsis* during recovery. Wild-type (A, C, and E) and *fab1* mutant (B, D, and F) plants were germinated and grown for 14 d at 22°C and then transferred to 2°C. After 21 d at 2°C, the plants were photographed (A and B) and transferred back to 22°C. The same plants were subsequently photographed after 2 d (C and D) and 4 d (E and F) at 22°C. White dots mark some of the leaves used to provide fluorescence data shown in Figure 6.

compositions (Hugly and Somerville, 1992; Murata et al., 1992; Wolter et al., 1992; Miquel et al., 1993). During the first 7 to 10 d at 2°C, we could not distinguish any differences in growth rate, photosynthetic fluorescence characteristics, or leaf cell ultrastructure between *fab1* plants and wild-type controls. After this relatively long lag period, F_v/F_m and the quantum yield of photosynthesis decreased steadily to reach a value of approximately 0 after 20 to 36 d

at 2°C. At the ultrastructural level, this decline in the photosynthetic capacity was accompanied first by extensive rearrangement of granal and stromal thylakoids and then by a complete dismantling of most of the chloroplasts observed in leaf mesophyll cells. Other cell structural features appeared to be maintained to a large extent during this period of chloroplast breakdown. The disappearance of chloroplast glycerolipids and Chl (Fig. 2) in the mutant

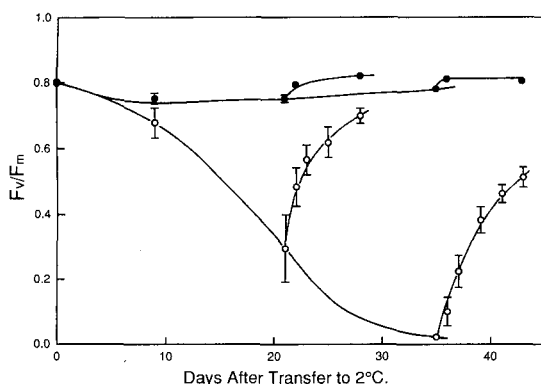


Figure 6. The F_v/F_m of PSII from wild-type (●) and mutant (○) *Arabidopsis* plants during growth at 22°C and subsequent recovery at 2°C. Plants were germinated, grown for 14 d at 22°C, and then transferred to 2°C. After 21 d at 2°C and again after 35 d, pots of wild-type and *fab1* plants were returned to 22°C and F_v/F_m was measured during the subsequent 7 d. The treatment that included 21 d at 2°C involved the same plants shown in Figure 5.

were rather modest but are nevertheless in qualitative agreement with the losses of thylakoid membranes that are evident in Figure 4.

The changes we observed appear to represent a type of autophagy—a regulated breakdown of cytoplasmic and organellar components observed in leaf tissues and cultured cells during periods of induced starvation (Wittenbach, 1978; Wittenbach et al., 1982; Journet et al., 1986). The process of autophagy is reasoned to be a regulated metabolic response that permits cells and tissues to endure a prolonged period of starvation, with the best possible chances for survival and growth once favorable nutrient conditions are reestablished. Consistent with this proposal, we observed a dramatic recovery of F_v/F_m and ϕ_{II} upon return of *fab1* plants to 22°C. Furthermore, even those leaves on *fab1* plants that remained chlorotic during recovery did not show any patches of necrosis and appeared to be composed of fully viable cells.

Our interpretation of these observations is that in the *fab1* mutant the alterations in chloroplast lipid composition relative to wild type disrupt long-term maintenance processes in the chloroplast, specifically at low temperatures. In transgenic tobacco plants, which like the *fab1* mutants have increased levels of thylakoid 16:0, damage at low temperature has been shown to be associated with a reduced capacity for recovery from short-term photoinhibition (Moon et al., 1995). Other mutations that result in reduced thylakoid unsaturation in higher plants or cyanobacteria also induce damage at low temperatures (Hugly and Somerville, 1992; Gombos et al., 1992; Kanervo et al., 1995).

The concept that relatively high levels of thylakoid unsaturation are necessary to allow adequate insertion and assembly of photosystem components at low temperature may provide the basis for a broad explanation of the several different phenotypes that have been described (Somerville, 1995). Whatever the mechanism, this disruption of maintenance processes in *fab1* plants eventually leads to a decrease in photosynthetic activity. When the starch and

Suc reserves of the plants are depleted, autophagic breakdown of the chloroplasts begins to sustain metabolic activity. Without a careful investigation of the timing of changes in net gas exchange, fluorescence characteristics, and chloroplast ultrastructure, it is not possible to definitively establish whether disruption of chloroplast ultrastructure is an autophagic response to reduced photosynthesis, as we suggest, or is instead the primary low-temperature lesion in *fab1* plants, which results secondarily in the reduction of photosynthetic function.

Indirect evidence to support the first interpretation comes from fluorescence experiments on the *fad2-2* mutant of *Arabidopsis*. In the *fad2* mutants the chloroplast fatty acid composition is substantially unaltered relative to the wild type, and it is likely that the effects of the mutation are mediated by the reduced levels of polyunsaturated fatty acids in extrachloroplast membranes of the cell (Miquel and Browse, 1992). The *fad2-2* mutants after transfer to low temperatures stop growing, gradually lose Chl, and develop necrotic patches on the leaves (Miquel et al., 1993). Nevertheless, when *fad2-2* plants were included in the experiment shown in Figure 6, the values of F_v/F_m (and also ϕ_{II}) measured on their leaves were within 5 to 10% of the values measured on the wild-type leaves even after 35 d at 2°C. By this time *fad2-2* leaves had lost 70 to 75% of their original Chl content (J.-M. Routaboul and J. Browse, unpublished data). This type of result is possible because F_v/F_m , and to a large extent ϕ_{II} , are density-independent measurements. Both fluorescence parameters reflect the state of those PSII centers that are absorbing and fluorescing light. Any photosynthetic machinery that has been dismantled or destroyed does not contribute to the measurement. We hypothesize that, in *fad2-2* plants, surviving PSII centers (and their associated antenna systems) remain functional, even as the number of centers declines as a secondary result of the disruption of cellular metabolism mediated by the altered lipid composition of extrachloroplast membranes. In this case, destruction of the PSII centers is not associated with a decline in F_v/F_m . In contrast, the effect of low temperature on the *fab1* mutant appears to be a primary reduction in the function of the PSII centers, which then triggers an autophagic response.

Dramatic Recovery of Photosynthetic Function upon Rewarming

When plants maintained at 2°C for 21 or 35 d were transferred back to 22°C, the recovery of F_v/F_m was rapid and extensive. Because the data for recovery in Figure 6 were collected by repeated measurements of the same leaves, they make it clear that severely damaged tissue on *fab1* plants retains a considerable capacity for recovery.

As discussed above, F_v/F_m is a density-independent measurement. For this reason, it is not completely clear whether the increasing F_v/F_m reflects a repair of damaged photosynthetic machinery in pre-existing cells of the leaf or the production of new cells in meristematic regions of the leaf lamella with the associated biogenesis of new chloroplasts. However, careful measurements of the length and width of individual leaves used in the experiment suggest

that an increase in leaf area of less than 10% accompanies the considerable regreening that is evident in Figure 5. It is, therefore, likely that at least some repair occurs in pre-existing cells, although the chlorosis of chilled leaves after 4 d of recovery (Fig. 5) indicates that this recovery is far from complete.

Comparison with *fad5*, a Second Mutant That Contains Increased 16:0

In *fab1* plants all of the individual membrane glycerolipids show some increase in 16:0 relative to the wild type (Wu et al., 1994). As a result, consideration of the *fab1* mutants alone has not allowed us to determine whether the general increase in saturated fatty acids produces the low-temperature phenotype or whether a more specific lipid change (such as the increase in the high-melting-point molecular species of PG) might be involved (Wu and Browse, 1995). However, another mutant of *Arabidopsis*, *fad5* (formerly *fadC*), also contains increased levels of 16:0 (Kunst et al., 1989a). The low-temperature phenotype of the *fad5* mutant is mild compared with that described here for the *fab1* line and appears in only tissue that develops after the time of transfer to low temperature (older leaves remain unaffected; Hugly and Somerville, 1992). Although young *fad5* plants become chlorotic and show a reduced growth rate relative to wild type at low temperatures, they are nevertheless able to flower and produce seed at temperatures that result in the death of *fab1* plants.

The *fab1* plants contain elevated levels of 16:0 as a result of reduced activity of 3-ketoacyl-acyl-carrier protein synthase II, the condensing enzyme that is the first step in converting 16:0 to 18:0 (Wu et al., 1994). The *fad5* mutants of *Arabidopsis* contain increased 16:0 in their leaf lipids because they are deficient in desaturation of 16:0 to Δ^7 -16:1 on chloroplast glycerolipids (Kunst et al., 1989a). The proportion of 16:0 in the total leaf lipids of the two lines is approximately the same (slightly higher in *fad5* than *fab1*), but analyses of individual leaf glycerolipids from the mutants reveal differences between the lines (Kunst et al., 1989a; Wu et al., 1994). The extrachloroplast phospholipids and digalactosyldiacylglycerol each show similar increases in 16:0 (relative to wild type) in both *fab1* and *fad5*. For MGD, the increase in 16:0 is very modest for *fab1* compared with *fad5*. In contrast, PG and SL show substantial increases in 16:0 content as a result of the *fab1* mutation, whereas these lipids are almost completely unaffected in the *fad5* plants.

These comparisons of the *fab1* and *fad5* lines indicate that an overall increase in leaf 16:0 may promote a mild, low-temperature phenotype, but the severe symptoms described here for the *fab1* mutant are probably caused by the increases in the 16:0 content of PG and possibly SL that are specific results of the *fab1* lesion. In PG the increased 16:0 is associated with the high-melting-point molecular species that contain only saturated or *trans*-unsaturated fatty acids (Wu and Browse, 1995). The involvement of the high-melting-point PG in mediating low-temperature damage of *fab1* plants is in broad agreement with other recent studies in which the fatty acid compositions of chloroplast lipids

were altered by transgenic techniques (Murata et al., 1992; Wolter et al., 1992; Moon et al., 1995). Even though these results suggest that increased 16:0 in PG (and possibly SL) results in a more severe, low-temperature phenotype than increased 16:0 in other membrane lipids, it is nevertheless entirely possible that the physiological effects of these and other reductions in membrane unsaturation are all mediated through inhibition of insertion or assembly of photosystem components in the thylakoid (Somerville, 1995), as discussed above.

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